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Diaporthe elaeagni-confertae and *D. fohaiensis* spp. nov. from Yunnan Province, China

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Abstract—Three *Diaporthe* strains were isolated from the leaf spots of diseased *Elaeagnus conferta* and *Lithocarpus fohaiensis* in Yunnan Province, China. Based on a combination of morphology and a five loci phylogeny (ITS, tub2, tef1-α, cal, and his3) indicated that these strains represented two new species, *Diaporthe elaeagni-confertae* and *D. fohaiensis*.

Key words—asexual *Ascomycota*, *Diaporthales, Diaporthaceae*, phylogeny, taxonomy

Introduction

Diaporthe Nitschke (*Diaporthaceae*, *Diaporthales*) was described by Nitschke (1870). Rossman & al. (2015) pointed out that the name *Diaporthe* has priority over *Phomopsis* (the name previously used for asexual morphs) in the context of the one fungus – one name initiative, and most accepted species have a *Diaporthe* name. *Diaporthe* is mainly characterised by immersed ascomata and erumpent pseudostroma with elongated perithecial necks; asci are unitunicate, clavate to cylindrical; ascospores are fusoid, ellipsoid to cylindrical, hyaline, biseriate to uniseriate in the ascus, sometimes with appendages (Udayanga & al. 2011; Senanayake & al. 2017, 2018). The asexual morph (*Phomopsis*) is mainly characterised by ostiolate conidiomata with cylindrical phialides that produce up to three forms of hyaline, aseptate conidia called α-conidia,

β-conidia, and γ-conidia (Udayanga & al. 2011; Gomes & al. 2013). The γ-conidia are rarely produced and difficult to observe (Gomes & al. 2013; Guarnaccia & Crous 2017; Guo & al. 2020). Most *Diaporthe* species have been reported as destructive plant pathogens, innocuous endophytes isolated from asymptomatic plant tissues, saprophytes that colonize and degrade dead leaves, bark, and twigs, or pathogenic fungi that cause health problems in humans and other mammals (Murali & al. 2006; Udayanga & al. 2012; Gomes & al. 2013; Ménard & al. 2014; Guarnaccia & al. 2016, 2018; Torres & al. 2016; Senanayake & al. 2018). Currently, of more than 1100 epithets listed in *Diaporthe* by Index Fungorum (23 October 2022), only one-fifth has been well-studied with ex-type cultures and supplementary DNA barcodes (Guo & al. 2020; Yang & al. 2020). For phylogenetic analysis of *Diaporthe* species, a five loci dataset (internal transcribed spacer [ITS] / beta-tubulin [tub2] / translation elongation factor 1-α [tef1-α] / calmodulin [cal] / histone H3 [his3]) is the optimal combination of species delimitation revealed by Santos & al. (2017).

Yunnan Province, located in southeastern China, has a unique geography with distinct climatic coniditions and abundant plant resources. Its ecological environment is conducive to the growth of unusual microbial species, which creates a higher species diversity than elsewhere in China. During our study of *Diaporthe* species in Yunnan Province, two new *Diaporthe* species were identified based on morphological characters and phylogenetic analysis.

Materials & methods

Isolates and Morphological analysis

Diseased leaves were collected from *Elaeagnus conferta* and *Lithocarpus fohaiensis* in Menghai county, Yunnan Province, China. Tissue pieces (5 × 5 mm) were taken from the margin of leaf lesions and surface-sterilized by consecutively immersing in 75% ethanol solution for 1 min, 5% sodium hypochlorite solution for 30 s, and then rinsing three times with sterile distilled water for 1 min. The pieces were dried with sterilized paper towels and then placed on potato dextrose agar (PDA) (Cai & al. 2009). All the PDA plates were incubated at 25°C for 2–4 d, and from which the colonies periphery were picked out and inoculated onto new PDA plates. Colonies photos in 7 d and 15 d were taken by digital camera (Powershot G7X mark II). Micromorphological characters were observed using Olympus SZX10 stereomicroscope and Olympus BX53 microscope, all fitted with Olympus DP80 high definition colour digital cameras to photo-document fungal structures. All fungal strains were stored in 10% sterilized glycerin at 4 °C for further studies. The studied specimens are deposited in the Herbarium of Plant Pathology, Shandong Agricultural University (HSAUP). Ex-type cultures are deposited in the Shandong Agricultural University Culture Collection

(SAUCC), Taian, Shandong Province, China. Taxonomic information of new species were submitted to MycoBank (http://www.mycobank.org).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from colonies grown on PDA, using the CTAB method (Guo & al. 2000). The five gene regions were amplified and sequenced with the following primer pairs: ITS with ITS4/ITS5 (White & al. 1990); tub2 with Bt2a/Bt2b (Glass & Donaldson 1995); tef1-α with EF1-728F/EF1-986R (Carbone & Kohn 1999); cal with CAL-228F/CAL-737R (Carbone & Kohn 1999); and his3 with CYLH3F/H3-1b (Glass & Donaldson 1995; Crous & al. 2004). PCR was performed using an Eppendorf Master Thermocycler (Hamburg, Germany). Amplification reactions were performed in a 25 µL reaction volume which contained 12.5 µL Green Taq Mix (Vazyme, Nanjing, China), 1 µL of each forward and reverse primer (10 µM) (Biosune, Shanghai, China), and 1 µL template genomic DNA in amplifier, and were adjusted with distilled deionized water to a total volume of 25 µL. PCR parameters were as follows: 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at a suitable temperature for 30 s, extension at 72°C for 1 min and a final elongation step at 72°C for 10 min. Annealing temperature for each gene was 55°C for ITS, 60°C for tub2, 52°C for tef1-α, 54°C for cal, and 57°C for his3. PCR products were estimated visually by staining with GelRed after 1% agarose gel electrophoresis. Sequencing was done bi-directionally by the Biosune Company Limited (Shanghai, China). Consensus sequences were obtained using MEGA 7.0 (Kumar & al. 2016). Sequences generated in this study were deposited in GenBank (Table 1).

Phylogenetic analyses

Novel sequences generated from our three strains in this study, and all available reference sequences of *Diaporthe* species were downloaded from NCBI's GenBank and alignments of the individual locus determined using MAFFT v. 7.110 (Katoh & al. 2019), and manually corrected where necessary. To establish the identity of the isolates at species level, phylogenetic analyses were conducted first individually for each locus (data not shown) and then as combined analyses of the five loci. Phylogenetic analyses were based on Maximum likelihood (ML) and Bayesian Inference (BI) for the multi-locus analyses. ML and BI were run on the CIPRES Science Gateway portal (https://www.phylo.org/; Miller & al. 2012) using RaxML v. 8.2.10 (Stamatakis 2014) and MrBayes v. 3.2.6 (Ronquist & Huelsenbeck 2003), respectively. For ML analyses the default parameters were used and BI was carried out using the rapid bootstrapping algorithm with the automatic halt option. Bayesian analyses included five parallel runs of 5,000,000 generations, with the stop rule option and a sampling frequency of 1000 generations. The burn-in fraction was set to 0.25 and posterior probabilities (PP) were determined from the remaining trees. The resulting trees were plotted using FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree) and edited with Adobe Illustrator CS v. 5.

Table 1. Species and sequences used in phylogenetic analyses. New sequences in bold. Isolates marked with "*" are ex-type or ex-epitype strains.

Species name	Voucher	Host/Substrare	Genbank accession number				
			ITS	tub2	tef1-α	cal	his3
*Diaporthe biguttulat*a	CGMCC 3.17248* = ZJUD 47	*Citrus limon*	KJ490582	KJ490403	KJ490461	—	KJ490524
	CGMCC 3.17249 = ZJUD 48	*Citrus limon*	KJ490583	KJ490404	KJ490462	—	KJ490525
D. brasiliensis	CBS 133183*	*Aspidosperma tomentosu*m	KC343042	KC344010	KC343768	KC343284	KC343526
D. caatingaensis	URM 7486* = CBS141542	*Tacinga inamoena*	KY085926	KY115600	KY115603	KY115597	KY115605
D. cinnamomi	CFCC 52569	*Cinnamomum* sp.	MH121504	MH121586	MH121546	—	MH121464
	CFCC 52570	*Cinnamomum* sp.	MH121505	MH121587	MH121547	—	MH121465
D. citriasiana	ZJUD81	*Citrus paradis*i	KJ490616	KJ490437	KJ490495	—	KJ490558
	ZJUD82	*Citrus paradisi*	KJ490617	KJ490438	KJ490496	—	KJ490559
D. discoidispora	ZJUD87 = CGMCC 3.17254	*Citrus sinensis*	KJ490622	KJ490443	KJ490501	—	KJ490564
D. elaeagni-confertae	**SAUCC194.47***	***Elaeagnus conferta***	**MT822575**	**MT855772**	**MT855888**	**MT855656**	**MT855544**
D. fohaiensis	**SAUCC194.113***	***Lithocarpus fohaiensis***	**MT822641**	**MT855838**	**MT855953**	**MT855720**	**MT855608**
	SAUCC194.115	***Lithocarpus fohaiensis***	**MT822643**	**MT855840**	**MT855955**	**MT855722**	**MT855610**
D. malorum	CAA752* = CBS 142383	*Malus domestica*	KY435643	KY435671	KY435630	KY435661	KY435651
	CAA740	*Malus domestica*	KY435642	KY435670	KY435629	KY435660	KY435650

Species name	Voucher	Host/Substrare	Genbank accession number				
			ITS	tub2	tef1-α	cal	his3
D. minusculata	CGMCC 3.20098*	on decaying branch	MT385957	MT424712	MT424692	—	—
	GZCC 19-0345	on decaying branch	MT797184	MT793038	MT793027	—	—
D. oxe	CBS 133186*	*Maytenus ilicifolia*	KC343164	KC344132	KC343890	KC343406	KC343648
	CBS 133187	*Maytenus ilicifolia*	KC343165	KC344133	KC343891	KC343407	KC343649
D. paranensis	CBS 133184*	*Maytenus ilicifolia*	KC343171	Kc344139	KC343897	KC343413	KC343655
D. siamensis	MFLUCC 10-0573a	*Dasymaschalon* sp.	JQ619879	JX275429	JX275393	JX197422	—
	MFLUCC 10-0573b	*Dasymaschalon* sp.	JQ619880	JX275430	JX275395	JX197423	—
D. tarchonanthi	CPC 37479 = CBS 146073	*Tarchonanthus littoralis*	MT223794	MT223733	—	—	MT223759
D. yunnanensis	CGMCC 3.18289* = LC6168	*Coffea* sp.	KX986796	KX999228	KX999188	KX999290	KX999267
	LC8106	*Coffea* sp.	KY491541	KY491561	KY491551	KY491571	—
	LC8107	*Coffea* sp.	KY491542	KY491562	KY491552	KY491572	—
Diaporthella corylina	CBS 121124	*Corylus* sp.	KC343004	KC343972	KC343730	KC343246	KC343488

Phylogentic results

Three *Diaporthe* strains isolated from plant hosts were sequenced. *Diaporthe* spp. was analysed by using multi-locus data from 26 isolates including *Diaporthella corylina* (CBS 121124) as outgroup. A total of 2658 characters including gaps were obtained in the phylogenetic analysis: ITS, 1–609; tub2, 610–1185; tef1-α, 1186–1601; cal, 1602–2141; and his3, 2142–2658. Of these characters, 1683 were constant, 365 were variable and parsimony-uninformative, and 610 were parsimony-informative. For the BI and ML analyses, GTR+I+G for ITS and his3, HKY+G for tub2 and cal, GTR+G for tef1-α were selected and incorporated into the analyses. The topology of ML tree confirmed the tree topologies obtained from the BI analyses, and therefore, only the ML tree is presented (Fig. 1).

ML bootstrap support (BS) ≥50% and Bayesian posterior probability (PP) ≥0.90 are shown as first and second position. The 26 strains were assigned to 16 species clades, based on the five gene loci phylogeny (Fig. 1). In this tree, our three new strains were assigned to two novel species, *Diaporthe elaeagni-confertae* and *D. fohaiensis*.

Taxonomy

Diaporthe elaeagni-confertae S.T. Huang, J.W. Xia, X.G. Zhang & W.X. Sun, **sp. nov.** Fig. 2

MB 839334

Differs from *Diaporthe siamensis* by its larger β-conidia; and from *D. fohaiensis* by its production of α-conidia and its shorter β-conidia.

Type: China, Yunnan Province, Menghai county, Nannuoshan, on infected leaves of *Elaeagnus conferta* Roxb. (*Elaeagnaceae*), 19 April 2019, S.T. Huang (**Holotype**, HSAUP194.47; ex-type living culture, SAUCC194.47; GenBank MT822575, MT855772, MT855888, MT855656, MT855544).

Etymology: refers to the host *Elaeagnus conferta*, on which the fungus was collected.

Colonies covering dish after 15 days in the dark at 25°C on PDA. Cottony with abundant aerial mycelium, and concentric zonation, both white on surface side and reverse. Conidiomata pycnidial, subglobose to globose, multi-pycnidia aggregated in groups, gray to black, erumpent, coated with white and grayish hyphae, thick-walled, exuding creamy conidial droplets from ostiole. Paraphyses not observed. Conidiophores hyaline, smooth, rarely septate, branched, densely aggregated, cylindrical, straight to slightly sinuous, 8.0–13.5 × 2.0–2.5 μm. Conidiogenous cells 12.5–15.5 × 1.5–2.2 μm, cylindrical, guttulate, terminal, tapering towards the apex. Alpha conidia, biguttulate or

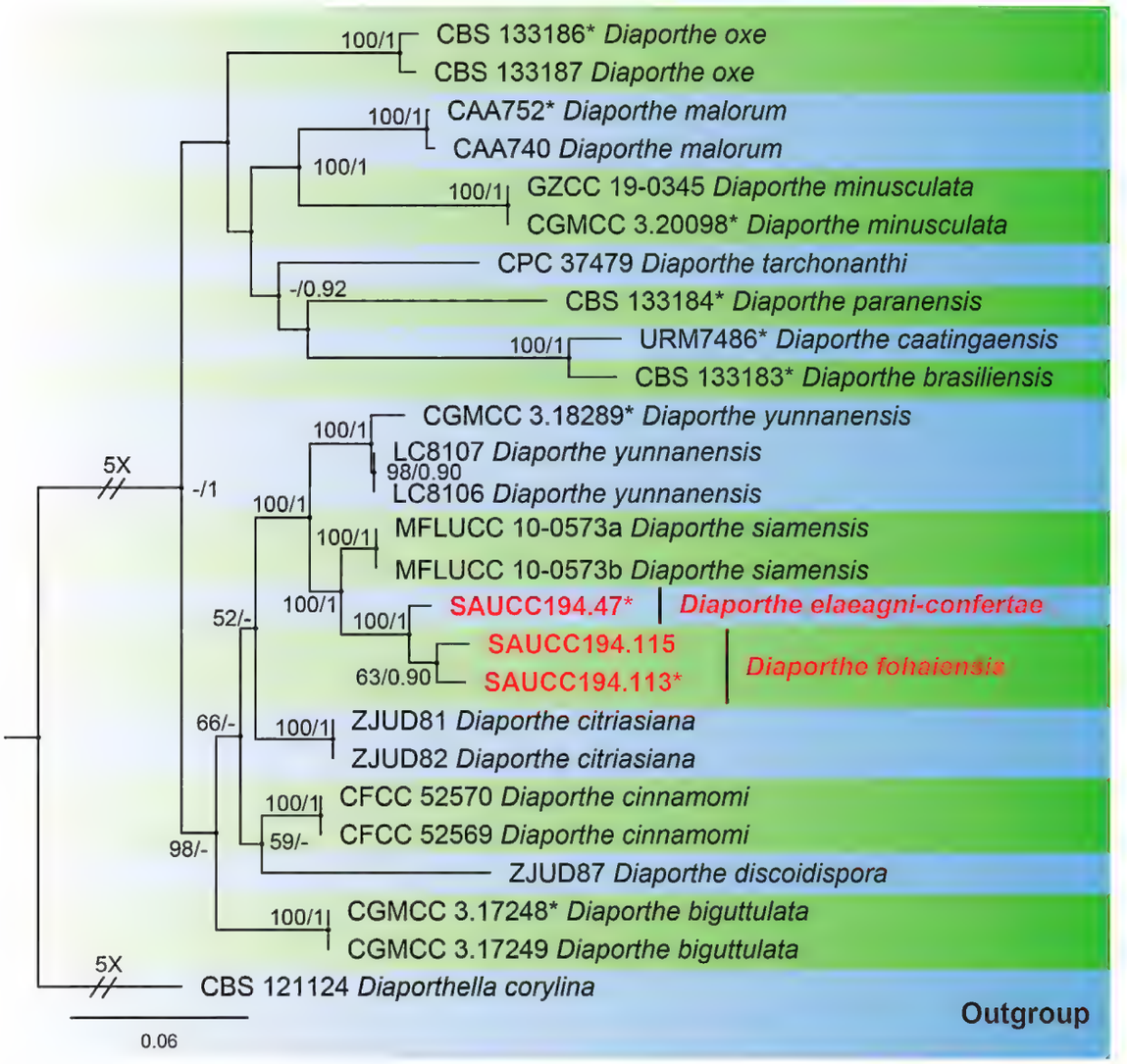

FIG. 1. Phylogram of *Diaporthe* based on combined ITS, tub2, tef1-α, cal, and his3 genes. Bootstrap support values are shown for ML ≥50% and BI ≥0.90. Strains marked with "*" are ex-type or ex-epitype. Our three new strains are in red. Some branches were shortened to fit them to the page – these are indicated by two diagonal lines accompanied by the length reduction factor.

eguttulate, hyaline, smooth, aseptate, ellipsoidal, oval, 4.5–6.5 × 1.8–2.8 µm (mean = 5.9 × 2.3 µm, n = 20). BETA CONIDIA abundant in culture, hyaline, aseptate, filiform, mostly curved through 0–90° at the apex, tapering towards the apex, base truncate, 29.5–36.0 × 1.0–1.2 µm (mean = 30.5 × 1.2 µm, n = 20). GAMMA CONIDIA not observed. Sexual morph: undetermined.

COMMENTS—Phylogenetic analysis of a combined five loci showed that *Diaporthe elaeagni-confertae* (strain SAUCC194.47) formed an independent clade (FIG. 1) and is phylogenetically distinct from *D. fohaiensis* and *D. siamensis* (Gao & al. 2016). This species can be easily distinguished from *D. siamensis* by 64 nucleotides difference in the concatenated alignment (16/491 in ITS;

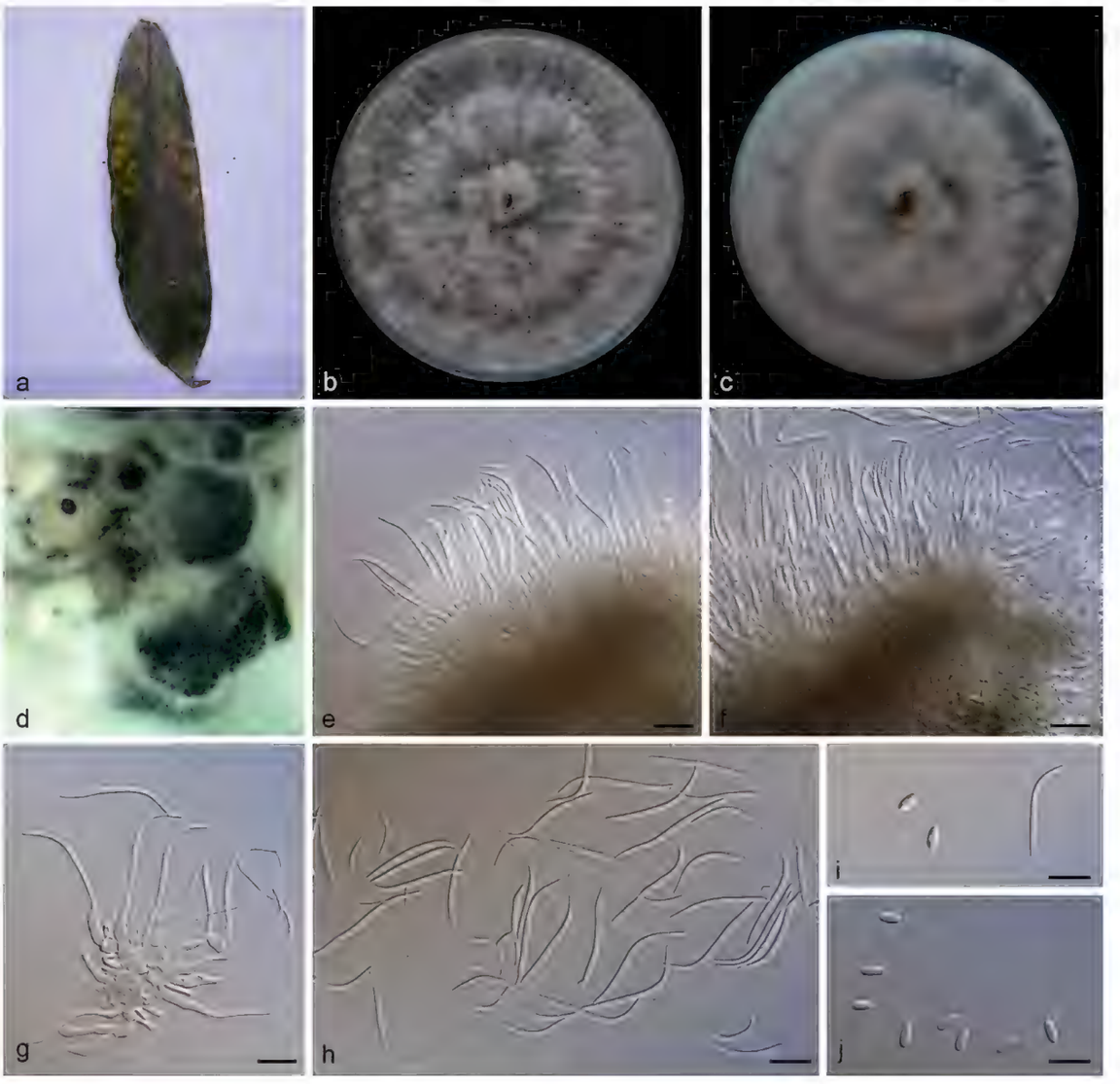

Fig. 2. *Diaporthe elaeagni-confertae* (ex-holotype, SAUCC194.47). a. Leaves of host plant; b. Surface of colony after 15 days on PDA; c. Reverse of colony after 15 days on PDA; d. Conidiomata sporulating on PDA margin; e–g. Conidiogenous cells with developing conidia; h. β-conidia; i. α-conidia and β-conidia; j. α-conidia. Scale bars: e–j = 10 μm.

8/446 in tub2; 24/346 in tef1-α; and 16/442 in cal); and from *D. fohaiensis* by 46 nucleotides difference concatenated alignment (10/556 in ITS; 9/499 in tub2; 4/340 in tef1-α; 16/467 in cal; and 7/444 in his3). Morphologically, *D. siamensis* differs by its smaller β-conidia (15.0–18.0 × 1.5–2.0 μm), and *D. fohaiensis* differs by its longer β-conidia (33–37.5 μm long) and its lack of α-conidia.

Diaporthe fohaiensis S.T. Huang, J.W. Xia, X.G. Zhang & W.X. Sun, **sp. nov.**

MB 839335 Fig. 3

Differs from *Diaporthe elaeagni-confertae* by its lack of α-conidia, its longer β-conidia, and its shorter conidiogenous cells.

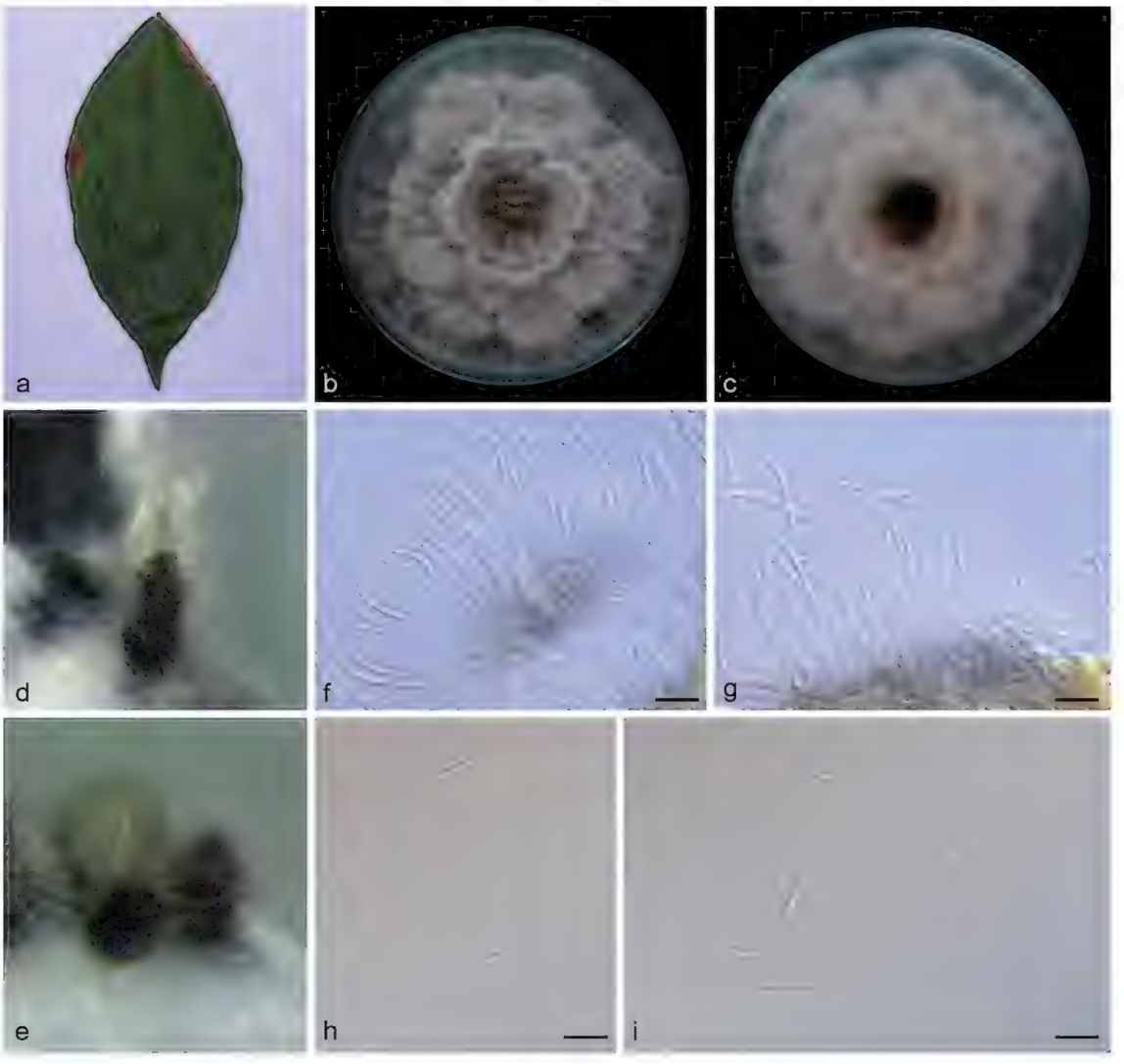

Fig. 3. *Diaporthe fohaiensis* (ex-holotype, SAUCC194.113). a. Leaves of host plant; b. Surface of colony after 15 days on PDA; c. Reverse of colony after 15 days on PDA; d, e. Conidiomata sporulating on PDA margin; f, g. Conidiogenous cells with developing conidia; h, i. β-conidia. Scale bars: f–i = 10 μm.

Type: China, Yunnan Province, Menghai county, Nannuoshan, on infected leaves of *Lithocarpus fohaiensis* (Hu) A. Camus (*Fagaceae*), 19 April 2019, S.T. Huang (**Holotype**, HSAUP194.113; ex-type living culture, SAUCC194.113; Genbank MT822641, MT855838, MT855953, MT855720, MT855608).

Etymology: refers to the epithet of the host *Lithocarpus fohaiensis* on which the fungus was collected.

Colonies covering dish after 15 days in the dark at 25°C on PDA. Cottony with abundant aerial mycelium, hyphae white on surface side, lobate in the margin, with pare brown concentric ring of dense hyphae on reverse side. Conidiomata pycnidial, subglobose to globose, scattered or multi-pycnidia

aggregated in groups, pale brown to black, erumpent, coated with white and gray hyphae, thick-walled, exuding creamy conidial droplets and spiral conidial cirrus from ostiole. Conidiophores hyaline, smooth, rarely septate, branched, densely aggregated, cylindrical, straight to slightly sinuous, 4.5–9.5 × 1.8–3.0 μm. Conidiogenous cells, cylindrical, terminal, rarely guttulate, tapering towards the apex, 7.8–11.5 × 1.5–2.5 μm. Alpha conidia and gamma conidia not observed. Beta conidia abundant in culture, hyaline, aseptate, filiform, mostly curved through 90–180° at the apex, tapering towards both ends, base truncate, 33–37.5 × 1.0–1.5 μm (mean = 35.5 × 1.3 μm, n = 20). Sexual morph: undetermined.

Additional specimen examined: **CHINA, Yunnan Province, Menghai county**, Nannuoshan, on diseased leaves of *Lithocarpus fohaiensis*, 19 April 2019, S.T. Huang (HSAUP194.115; living culture SAUCC194.115; Genbank MT822643, MT855840, MT855955, MT855722, MT855610).

Comments—Phylogenetic analysis of a combined five loci showed that *Diaporthe fohaiensis* (strains SAUCC194.113 and SAUCC194.115) formed an independent clade (Fig. 1), and is phylogenetically distinct from *D. elaeagni-confertae*. This species can be easily distinguished from *D. elaeagni-confertae* by 46 nucleotides difference in the concatenated alignment (10/556 in ITS; 9/499 in tub2; 4/340 in tef1-α; 16/467 in cal; and 7/444 in his3). Morphologically, *D. fohaiensis* differs from *D. elaeagni-confertae* which has α-conidia, shorter β-conidia (29.5–36.0 μm long), and longer conidiogenous cells (12.5–15.5 μm long).

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